Remarks:

Please find remarks directed towards the individual points of the examiner's detailed action of the 02/18/2005 office action itemized below:

Disclosure Objections:

1.) The reference to US Patent 5,528,453 refers to an incorrect inventor.

To correct this, all of the patent references have been changed to those which more adequately address this application. The information disclosure statement (USTPO PTO/SB/08B or 1449B) has been filled out, and is included with the revised application. The new patent references are included, with correct information. A substitute specification is enclosed, with a more complete discussion of the prior art in regards to these references.

Claims Objections:

The examiner had noted a number of problems with the claims. Since the correction of these would have produced an undesirably marked up presentation, all previous claims have been canceled, and re-stated as new claims. In addition, the re-drafting of the old claims produced dependent claims which were not grouped near their respective independent claims. In order for the claims to be grouped together in a logical manner, a re-stating all claims as new was required. The new claims precisely parallel the old in content.

An itemized list of corrections to the stated claim objections follows:

2.) Incorrect multiple dependent references were used.

To correct this, all use of multiple dependent claims has been eliminated.

3.) A number of minor informalities in the claims were noted.

To correct this, the punctuation of all claims has been changed so that there is only one capital letter at the beginning, and one period at the end. All typographical errors have been corrected. All vague references such as "any other", "other form of", "any other suitable", "other based", or "but not limited to", hereafter referred to", have been eliminated and replaced with normal patent language. The use of abbreviations is now consistent.

Claims Rejections

4.) The specification does not present a clear concise method of the invention and the manner of using it.

To correct this, a substitute specification is submitted, to correct this and a number of problems. The applicant attests that the substitute specification contains no new matter. Mush of the deleted material (strikethrough) and new material (underlined) shown in the

marked copy only reflect the re-arrangement of previously existing text. The previous brief description of the drawings was not truly brief. This section has been changed so that all descriptions of drawings consist of one to two sentences. The previous detailed description of the invention did not refer to the drawings in the extent that it should. To correct this, the previous in depth description of the drawings has been added to the detailed description of the invention. References to the drawings are now made throughout the detailed description of the invention, and fit in with the detailed description to more adequately explain the invention. The substitute specification contains new headings which more adequately define certain procedures, and point out which figures each section of the specification refers to. These headings contain terms which can now be referred to in the claims, to mark which section of the specification that the claims refer to. For instance, the term "cation immobilization" is introduced to refer to the procedure where chlorin *e*6-transferrin is further purified, so that this procedure can be referred to in claim 45. These new headings do not add new matter to the specification. In the old specification, the term sepharose was used. This was an error as it should have indicated sephadex. The use of the proper name Bio-Rad to describe the Bio-Rad multi imager was changed to a non-proper name format, where the term CCD camera equipped imager was used.

5.) Claims 25-31 contain subject matter which was not in the specification. The use of "coupling agent modified chlorin e6" is objected to.

This has been changed to "activated chlorin e6", in the new independent claim 43 [c]. This term was referred to in the original specification:

> The conjugation of chorin e6 to proteins usually occurs in solution with compounds such as EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) or cyclohexyl-3(2-morpholinoethyl) carbodiimide being present to **activate chlorin e6** carboxyl groups to amine-reactive entities (Akhlynina et. al., 1995; Bachor et. al., 1991). With EDC, chorin e6 carboxyl groups form *0*-acylisourea intermediates for their conjugation to protein primary amines.

This "activated chlorin e6", is then defined as chlorin e6 acted on by EDC, which is described in the specification. The claims have been re-written so that each procedure step defines the specific agent to be used, as defined in the specification. This was done by the use of wherein clauses to identify these agents.

6.) the mention of "coupling agents" is imprecise as any agents other than the specific one used could only be verified by persons skilled in the prior art, through undue experimentation.

This term has been replaced with "activating compounds" in claim 43 [c], and then is identified, in that same claim, as EDC, thereby referring to the procedure in the specification. It is thought that the use of the term "activating compounds" in this claim

is referred to in the specification, through the use of the term "to activate chlorin e6", the specification.

7.) The specification is enabling for iron saturated human transferrin only, and not any other transferrins that are mentioned in the claims.

The claim stating the use of transferrin, claim 43[b], has been changed to one where the human holo transferrin stated in the specification is used: "preparing a transferrin solution, by using a process comprising one wherein a transferrin is dissolved in said buffer, wherein said transferrin is comprising human holo-transferrin".

8.) and 9.) Indicate that the subject matter of the invention was not distinctly claimed.

With the re-organization of the specification and the claims, it is thought now that the method of conjugation using immobilization and a detergent in the reaction is the method claim that is now apparent, producing a product with bona-fide transferrin action, yet while possessing light-killing action. Also, the use of this particular product, made in this way, as stated in the use claims, is apparent.

10.) The major independent claim is indefinite in regards to step D.

This claim has been re-drafted as claim 43, where it uses correct language and is more clear. The indefinite step D has been re-stated as claim 44.

11.) The major independent claim uses improper terminology to refer to previous steps.

This claim has been re-drafted as claim 43, where it uses correct language in referring to previous steps .

12.) The solution of choice in claim 25 is indefinite.

This has been changed to "solution", in a general referral to any solution that is desired by the user. This is then further defined as the buffer by the use of the "wherein said solution is comprised of said buffer" phrase.

13.) The use of the phrase A "is", "but not limited to" B is objected to as improperly narrowing the claims.

The use of this phrase has been eliminated. The use of "comprises" , "selected from a group", etc. are now used throughout the claims so that proper patent language is used.

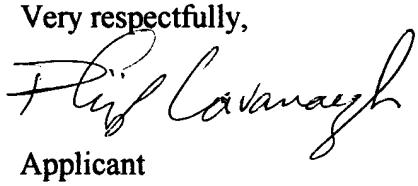
14.) The claims are narrative and contain indefinite language.

All claims have been re-written with proper punctuation, with a flow from broad to narrow, the use of patent language, etc., so as to more accurately define the invention.

Request for constructive assistance

The applicant has amended the specification, and claims so that they are proper and define a novel non-obvious method. If this application is not believed to be in condition for allowance, the applicant respectfully requests the constructive assistance and suggestions of the examiner pursuant to MPEP 2173.02 and 707.07(j), in order so that the applicant can submit an allowable application as soon as possible.

Very respectfully,



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In the Specification: A Marked-Up version of the substitute specification follows.

TITLE OF INVENTION:

SYNTHESIS, AND PHOTODYNAMIC THERAPY-MEDIATED ANTI-CANCER, AND OTHER USES OF CHORIN *E*6-TRANSFERRIN.

CROSS REFERENCE TO RELATED APPLICATIONS: None

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT: This invention was not directly supported by any federally sponsored research.

REFERENCE TO SEQUENCE LISTING, TABLES, OR COMPUTER PROGRAM LISTINGS: None

BACKGROUND OF THE INVENTION:

Rapidly growing cells require continuous intracellular iron transport in order to divide. Free iron, or iron salts, are absent in biological systems as iron salts can catalyze many un-favorable reactions (Conrad and Umbreit, 2000). Therefore, all iron delivery, storage, and transport in cells and higher organisms occurs while the iron is complexed to proteins. The major circulating iron transport protein is transferrin (Tf), which exists in blood at levels of 200 - 400 mg/100 ml (Ponka and Richardson, 1998). Each transferrin

protein binds and transports two atoms of iron. To accomplish iron internalization, cells express transferrin receptors (TfR; Testa, et. al., 1993; Ponka, et. al., 1998; Ponka and Lok, 1999) on their surface. These receptors interact with transferrin and two iron-saturated transferrins bind to one TfR. This TfR-Tf complex is internalized into the cell and the complexed iron is delivered to needed sites. Most tumor cells exhibit rapid growth rates and therefore internalize copious quantities of iron and express high levels of transferrin receptors (Gatter et. al., 1983; Niitsu et. al., 1987). Quiescent normal adult cells express little or no TfR (Gatter et. al., 1983; Tani et. al., 2000; Juhlin, 1989; Niitsu et. al., 1987) . Therefore, in many tissue areas, if a tumor exists, the only site of high TfR expression will be associated with the tumor cells. The expression of TfR in human tumor cells has been found to correlate with tumor grade, stage, progression, and metastasis. This has been seen in breast carcinomas (Wrba et. al., 1986), bladder transitional cell carcinomas (Seymour, et. al., 1987), and malignant melanoma (Van Muijen et. al., 1990). In addition, high levels of TfR have been observed in a metastatic lesion of a maxillary neoplasm, but not in the parental tumor (Yoda et. al., 1994),and the expression of TfR was higher in a human melanoma line selected for metastatic capability in nude mice than in the poorly metastatic tumor cells of the parental population (Van Muijen, et. al., 1991). In other studies, growth response to Tf was seen to correlate with metastatic progression in the B16 melanoma (Stackpole et. al., 1994) and Tf was identified as the major bone-marrow derived mitogen for bone-marrow metastasizing prostatic carcinoma cells (Rossi et. al., 1992). We have found that tumor cell expression of TfR can correlate with the metastatic ability of certain tumor cells

(Cavanaugh and Nicolson, 1991, 1998; Cavanaugh et. al., 1999), which indicates that heightened TfR expression can be associated with the more aggressive tumor cell types.

Therapy against cancer is ideal when cancer cells are specifically killed while normal cells are left largely intact. Furthermore, an ideal treatment is achieved when cell killing occurs only at the site of the tumor and any non-specific killing at other sites is avoided entirely. To achieve these ends, researchers designing anti-cancer therapies will direct cancer cell killing agents at cell components which are novel to cancer cells or are present at much greater numbers on cancer cells than on normal cells. Various toxin-conjugated or radioactive antibodies directed towards antigens expressed only on the surface of cancer cells have been produced and tested (Hudson, 1999; Scott and Welt, 1997). Strategies to combat cancer using reagents directed at the transferrin/TfR system are currently being explored, and these are most successful when used to treat tumors of hematopoetic origin (Elliot et. al., 1988; Kemp et. al., 1992, 1995; Kovar et. al., 1995). The problem with any agent of this nature is that they can act, albeit to a lesser degree, on normal cells nearby and distant from the tumor site, causing side effects. To circumvent the latter problem, treatments have been devised which attack cancer only at the site of the tumor. If a pre-toxin could be specifically delivered to the TfR, and could furthermore be specifically activated to the toxin state at a certain site, then a tumor cell specific, site specific killing of tumor cells could be achieved. If at the same time, the pre-toxin remained non-toxic at other sites where the activation was not performed, then side effects could be avoided.

Photodynamic therapy (PDT) is an anti-cancer strategy that has been the subject

of intensive study in recent years (Hsi et. al., 1999). The idea is to deliver to a tumor site a an inactive toxin which is then activated to a cell-killing toxin by exposure to light. Site-specific light irradiation causes site-specific cell killing. A number of different compounds which become toxic when impinged upon by light have been developed (Hsi et. al., 1999). These compounds have been conjugated to various proteins (Akhlynina et. al., 1995; Donald et. al., 1991; Gijsens and De Witte, 2000; Del Governatore et. al., 2000) or covalently linked to other molecules (Katsudemi et. al., 1994; Bachor et. al., 1991), to create a complex that when delivered *in vivo*, will produce a tumoricidal effect, when the tumor area is irradiated with light. One of the more useful PDT agents is chlorin *e*6, a nettle-derived porphyrin which is rendered toxic by irradiation with visible light.

We sought to conjugate transferrin with chlorin *e*6 , to develop an anti-cancer PDT agent which would exploit the high affinity of tumor cells for transferrin and the site-specific nature of PDT. The conjugation of chorin e6 to proteins usually occurs in solution with compounds such as EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) or cyclohexyl-3(2-morpholinoethyl) carbodiimide being present to activate chlorin e6 carbonyl groups to amine-reactive entities (Akhlynina et. al., 1995; Bachor et. al., 1991). With EDC, chorin e6 carboxyl groups form *O*-acylisourea intermediates for their conjugation to protein primary amines. Typically, once reactions are complete, conjugated proteins are separated from un-reacted intermediate and chlorin *e*6 by gel filtration. A number of these procedures were used to conjugate chorin *e*6 to transferrin with apparent success at conjugate formation, however the conjugate made using these methods consistently displayed none of transferrin's usual growth stimulating

activity on a particular target cell line. When conjugation using EDC was performed after immobilization of Tf to QAE-sephadex, biological activity of the ligand was maintained. The conjugated protein could be released from the gel by high salt only if a detergent such as CHAPS was present. Tf conjugated with chorin e6 in this fashion displayed cell growth-promoting activity, TfR binding activity, and displayed potent light-dependent killing of tumor cells in culture. As such, this patent and the invention is for this novel method for the conjugation of proteins to chorin *e*6, and for the subsequent use of this conjugate as a tumor-specific, tumor site-activatable, anti-cancer agent.

DISCUSSION OF THE PRIOR ART:

Transferrin has been suggested as a delivery vehicle for anticancer drugs (Singh, 1999) and non-chlorin *e*6 PDT conjugates of transferrin have been produced (Hamblin and Newman, 1994). However, follow-up studies and extensive *in vitro* or *in vivo* work with the latter have been lacking.

In U.S. patent 4,522,750, a procedure is described for the linking of transferrin to alkaloids. The procedure is similar to this invention, except that the transferrin is not immobilized first. The conjugate is an alkaloid and not a photodynamic agent. The compounds were tested for their ability to decrease leukemia growth in mice, where an effect was seen. However, no mention of the actual testing of the conjugates in regards to their acting through a bona fide transferrin/transferrin receptor mechanism is mentioned.

In U.S. patent 4,590,001 the binding of platinum to transferrin for use as an anti-cancer agent is described. The binding procedure and the toxic compound are different

than the ones mentioned in this application.

In U.S. patent 5,876,989, the use of a three component system for delivering photosensitizers into cells is mentioned. This consists of a photodynamic therapy agent, a molecule of interest, and a carrier molecule. The molecule of interest could be transferrin. The photodynamic therapy agent could be a chlorin. The showing of a forming of a conjugate of these is not made: they are administered together, but not as a linked conjugate.

In U.S. patent 5,906,977, the use of an inflammatory disease treating complex consisting of an active substance, a linker, and a carrier is discussed. The active substance could be a photodynamically active compound. The carrier could be transferrin. The method of conjugate synthesis is different than that here. The display of a biologically active transferrin conjugate is not shown. The use as an anti cancer agent is not discussed.

In U.S. patent 6,500,800, the use of a five component complex for causing damage to cells by photodynamic therapy is shown. The conjugates differ from the one here since in the current application, the carrier protein is missing. Also, the method of conjugation is different. Transferrin is mentioned as being a potential ligand, but the actual synthesis and testing of a transferrin-based conjugate is not shown.

In U.S. Patent 6,610,298, the use of photodynamic agent conjugates for treating mycobacterial infections is shown. The conjugates consist of a photosensitizer and a liposomal targeting moiety. The photosensitizer could be a chlorin. The targeting moiety could be transferrin. The conjugation method used does not involve immobilization of

the protein prior to conjugation. The actual use of a transferrin conjugate is not presented. The use of these conjugates as an anti cancer agent is not discussed.

U.S. Patent 6,812,209 is for conjugates of active compounds with native proteins. The active compound could be a photodynamic agent. The possibility that the protein could be transferrin is mentioned in the detailed description. The conjugation method used does not involve immobilization of the protein prior to conjugation. This invention covers a whole host of protein-based therapeutic reagents wherein the protein is conjugated with a chemotherapeutic or photodynamic agent. However, the specific protein mentioned in the claims is albumin.

~~BACKGROUND REFERENCES:~~

~~U.S. Patent Documents:~~

~~5,528,453~~	~~November, 1993~~	~~Koopecek, et. al.~~
~~5,876,989~~	~~March, 1999~~	~~Berg, et. al.~~
~~5,976,535~~	~~November, 1999~~	~~Fritzberg, et. al.~~
~~6,107,466~~	~~August, 2000~~	~~Hasan, et. al.~~

~~Specific Documents:~~

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BRIEF SUMMARY OF THE INVENTION:

Human iron-saturated transferrin was bound to quaternary-amino ethyl (QAE) sephadex in a buffer of 25 mM sodium phosphate, pH 7.2, containing 2 mM of the detergent CHAPS (PB/CHAPS buffer). The gel was washed free of unbound transferrin and was reacted directly with 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide

hydrochloride (EDC) and the porphyrin chlorin e6, in the same buffer. Or, chorin e6 was reacted with EDC in a separate vessel, in the PB/CHAPS buffer, and un-reacted chorin e6 removed from the mixture by adsorption to quaternary aminoethyl (QAE)-sephadex, all in PB/CHAPS. This latter soluble EDC-modified chlorin e6 was added to the immobilized transferrin to produce the immobilized conjugate. In either case, the transferrin was conjugated while bound to the gel and was washed free of un-reacted soluble conjugation components. The conjugate was then released from the gel by treatment with PB/CHAPS containing 0.5 M NaCl. The conjugate was dialyzed against PB for further use.

The conjugate was first shown to retain transferrin's growth promoting activity on the rat MTLn3 tumor line, in a low serum growth assay. The conjugate was then tested for its ability to compete with FITC-transferrin for binding to the transferrin receptor, using a western blot-mediated ligand binding assay. The conjugate was seen to possess an altered migratory pattern when analyzed by native gel electrophoresis. Finally, the conjugate was seen to kill tissue cultured tumor cells in a light-exposure dependent fashion. This killing effect was not evident in the absence of light or when excess un-conjugated transferrin was present, indicating a specific effect. Chlorin e6-transferrin prepared in this manner retains biological activity and is a candidate for use as a photodynamic therapy treatment of cancer and other disorders.

The invention presents a novel method for the conjugation of a porphyrin to a protein, in particular, the conjugation of chlorin e6 to transferrin. This results in the formation of a relatively tumor-specific ligand which possesses cell killing activity when

activated by photodynamic therapy. Although the use of transferrin as an anti-tumor photodynamic therapy agent has been discussed by others, the use of chlorin e6, the use of this conjugation technique, and an illustration of putative effect as presented here is not evident in the scientific or patent literature.

5

BRIEF DESCRIPTION OF THE DRAWINGS:

Figure 1 shows a schematic of chorin e6, and a schematic of the reaction of chlorin e6, EDC, and transferrin.

Figure 2 shows the effect of chorin e6 on the growth of Rat MTLn3 mammary

10 adenocarcinoma cells.

Figure 3 shows the native gel electrophoretic migration pattern of chlorin e6-transferrin (Ce6-Tf), in comparison to that of un-altered transferrin..

Figure 4 shows the competition of FITC-Tf binding to cell surfaces by Ce6-Tf.

Figure 5 shows the light-dependent killing of rat MTLn3 mammary adenocarcinoma

15 cells by Ce6-Tf.

Figure 6 shows the light-dependent killing of MTLn3 and NRK cells by Ce6-Tf.

Figure 7 shows the light-dependent killing of Human MCF7 breast cancer cells by Ce6-Tf.

Figure 8 shows the effect of chlorin e6 (Ce6) alone on the viability of Rat MTLn3

20 cells.

~~Figure 1. A: Schematic of chorin e6. B: Schematic of the reaction of chlorin e6, EDC, and transferrin.~~

~~Figure 2. Effect of chorin e6 on the growth of Rat MTLn3 mammary adenocarcinoma cells. Cells were plated at 2,000 cells/well in 96 well plates in ∝MEM containing 5% FBS. One day after plating, media was changed to ∝MEM containing 0.3% FBS. Increasing levels of human holo-Tf (Native Tf) or human Ce6-Tf (both in PBS) were added to respective wells, in the amount indicated. Four days later, cells were quantitated using a crystal violet stain assay, where A590 correlates with cell number. A: an image of the crystal violet stained plate used in the assay is shown. B: A plot of the absorbances from A. Cell number and is seen to rise as the cells are exposed to increasing levels of native human Tf. A similar, albeit slightly lower rise was seen with Ce6-Tf, indicating intact biological activity in the latter.~~

~~Figure 3. Native gel electrophoretic analysis of Ce6-Tf. 10 µg quantities of all proteins listed were treated, loaded, and run out using the native gel system. The gel was fixed and stained with Coomassie blue. The results indicate a greater mobility of chlorin e6-transferrin (lanes 5 and 6) when compared to native transferrin (lane 4).~~

~~Figure 4. Competition of FITC-Tf binding to cell surfaces by Ce6-Tf. Transferrin solvent, human Ce6-Tf, or native human Tf were added to Rat MTLn3 mammary adenocarcinoma cell monolayers equilibrated to 4° C. The final concentration of both~~

~~transferrins was 1mg/ml. FITC- human Tf was then added to all wells at 100 µg/ml. After a 2h incubation, cells were washed, lysed, electrophoresed, blotted, and examined for FITC content by incubation with anti-FITC and an HRP-conjugated second antibody, followed by ECL. A strong band at 70,000 Kd was seen from cell lysates which received FITC-Tf only (lanes 4 and 5), indicating FITC-Tf binding to the cells. Both Ce6-Tf (lanes 6 and) and native Tf (lane 8) competed out the FITC-Tf as indicated by the absence of any FITC signal in lysates from cells treated with either. Lanes 1-3 were loaded with known amounts of pure FITC-Tf, for standardization. An image of the ECL X-ray film is shown. The results indicate functional binding of Ce6-Tf to the transferrin receptor.~~

~~Figure 5. Light-dependent killing of rat MTLn3 mammary adenocarcinoma cells by Ce6-Tf. This was a continuous exposure, serum-free assay performed using protocol A described in the cell killing section. Cells were plated in 24-well plates and grown to confluency in ∝MEM containing 5% v/v FBS. On day one, media was changed to ∝MEM only and increasing levels of Ce6-Tf were added to test wells to a final concentration from 1.25 to 5.0 µg/ml. Native Tf was added to control wells at 5.0 µg/ml. On days 2, 3, and 4, cells were exposed to light from an X-ray film box for 15 min. Media and all Tf was changed each day. On day five, all cells were quantitated using the crystal violet stain assay. Images of the stained plates are shown in A. Stained cell numbers were evaluated using a Bio-Rad Multi-imager. The results of image analysis are shown in B, where ODU/mm2 correlates with cell number. Results indicate a light-~~

~~dependent killing as plates kept in the dark during the process displayed no loss of cell numbers.~~

~~Figure 6. Light-dependent killing of MTLn3 and NRK cells by Ce6-Tf. This was a one-day exposure, serum-containing assay performed using protocol B described in the cell killing section. Cells were plated in 24-well plates and grown to confluency. On day one, media was changed and increasing levels of Ce6-Tf were added to test wells to a final concentration from 7.5 to 30 ug/ml. Native Tf was added to control wells at 30ug/ml. On day 2, media was changed to that without added Ce6-Tf or Tf. On days 2, 3, and 4, cells were exposed to light from an X-ray film box for 15 min. Media was changed each day. On day five, all cells were fixed, stained, and quantitated using the crystal violet stain assay. Stained cell numbers were evaluated using a Bio-Rad Multi-imager. Images of the stained plates are shown in A and B. The results of image analysis are shown in C and D, where ODU/mm2 correlates with cell number. Results indicate a light-dependent killing as plates maintained in the dark during the process displayed no loss of cell numbers. The MTLn3 cell line was more susceptible to the effects of the Ce6-Tf as it showed a decrease in cell numbers at the 15 ug/ml dose whereas the normal NRK line did not.~~

~~Figure 7. Light-dependent killing of Human MCF7 breast cancer cells by Ce6-Tf. This was a one-day exposure, serum-containing assay performed using protocol B described in the cell killing section. Cells were plated in 24-well plates and grown to confluency. On~~

~~day one, media was changed and increasing levels of Ce6-Tf were added to test wells to a final concentration from 7.5 to 30 ug/ml. Native Tf was added to control wells at 30ug/ml. On day 2, media in all wells was changed to that without added Ce6-Tf or Tf. On days 2, 3, and 4, cells were exposed to light from an X-ray film box for 15 min. Media was changed each day. On day five, all cells were fixed, stained, and quantitated using the crystal violet stain assay. Stained cell numbers were evaluated using a Bio-Rad Multi-imager. Images of the stained plates are shown in A. The results of image analysis are shown in B, where ODU/mm2 correlates with cell number. Results indicate a light-dependent killing as plates kept in the dark during the process displayed no loss of cell numbers. As with the rat lines studied previously, this human line was also shown to be susceptible to a combination of Ce6-Tf and light.~~

~~Figure 8. A: Effect of Ce6 alone on the viability of Rat MTLn3 cells. Cells were tested as per method B outlined in the cell killing procedure description. Confluent cells in αMEM containing 5% FBS were exposed to the indicated concentrations of Ce6, Ce6-Tf, or Tf alone. One day later, media was changed to that without added Ce6-Tf or Tf, and all cells were exposed to light for 15 min. This was repeated on days two and three. Cells were then fixed and stained with Coomassie blue. An image of the stained wells is shown. The results indicate that Ce6 alone had no cell killing effect. B: Effect of excess Tf on the killing effect of Ce6-Tf. Cells were set up similarly as above, except that treatments consisted of Ce6-Tf, or Ce6-Tf in conjunction with 500 or 1,000 µg/ml native Tf. Light exposure, media changes, and cell staining were carried out as in A. An image~~

~~of the stained wells is shown. The results indicate that excess native Tf diminished the killing effect of Ce6-Tf, indicating that the latter acts through a Tf-specific process.~~

DETAILED DESCRIPTION OF THE INVENTION:

SYNTHESIS OF CHLORIN e6-TRANSFERRIN, FIGURE 1:

The positively charged matrix QAE sephadex A-50 was hydrated fully in water at a ratio of 1:100 (gel: water; w: v). The suspension was centrifuged at 1,000 X g for 5 min and the gel pellet equilibrated in 50 volumes of phosphate buffer (PB; 20 mM Na2HPO4, pH adjusted to 7.4 with KH2PO4). The gel was re-centrifuged and equilibrated in 10 volumes of phosphate buffer containing 2 mM CHAPS (3-[(3-cholidamidopropyl) dimethylammonio]-1-propane-sulfonate; buffer = PB/CHAPS). This was centrifuged at 1,000 X g for 5 min and the gel maintained in a minimal volume of PB/CHAPS. Iron-saturated human transferrin (Sigma Chemical, St. Louis, MO) was dissolved in PB/CHAPS to a concentration of 10 mg/ml. To 2 ml of Tf solution was added 0.5 ml of equilibrated QAE-sephadex slurry. This was mixed slowly by rocking for 30 min. The gel was washed three times by suspension in and centrifugation from 25 ml PB/CHAPS. To ensure saturation of the gel, the transferrin binding process was repeated. To make the conjugate, to 0.5 ml of QAE-sephadex-Tf was added 0.5 ml of a 2 mg/ml chlorin *e6* solution (Porphyrin products; Logan, Utah), dissolved in PB/CHAPS. The structure of chlorin *e6* is shown in Figure 1A. To this was added 150 uL of 10 mg/ml EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride, dissolved in water. The structure of EDC is shown at the top of Figure 1B. The structure of the

reaction product of EDC and chlorin *e*6 is shown in the middle left of Figure 1B. This mixture was rocked for 20 min at 25° C. The mixture was centrifuged at 1,000 X g for 5 min and the supernatant removed. To ensure complete conjugation, an additional 0.5 ml of chlorin *e*6 and 150 uL of EDC were added to the gel. The gel mixture was rocked again at 25° C for 25 min and the gel was washed four times by repeated suspension in and centrifugation (1,000 X g for 5 min) from 25 ml of PB/CHAPS. The bottom of Figure 1B shows a schematic of the chlorin *e*6-transferrin conjugate. To elute the conjugated Tf, the gel was suspended in 1ml PB/CHAPS containing 0.5 M NaCl. This was rocked for 20 min at 25° C, centrifuged at 1,000 X g for 5 min, and the supernatant collected. The elution step was repeated on the gel pellet and the supernatants pooled. The pooled chlorin e6-transferrin was dialyzed overnight at 4° C against 4L of PB containing 0.15 M NaCl.

ADDITIONAL PROCEDURE FOR THE PRELIMINARY PREPARATION OF EDC-CHORIN *e*6, FIGURE 1:

Chlorin e6 is dissolved at 1 mg/ml in 25 mM sodium phosphate, pH 7.2 containing 2 mM CHAPS. One tenth volume of 10 mg/ml EDC (in water) is added and allowed to react with the chlorin e6 at room temperature for 20 minutes. Figure 1A shows a schematic of chlorin e6 and Figure 1B displays the formula for EDC. The structure of the reaction product of EDC and chlorin *e*6 is shown in the middle left of Figure 1B. Also present in this mixture is un-reacted chlorin e6. This mixture is combined with an equal volume of a 50% (vol/vol) slurry of QAE-sephadex suspended in and equilibrated

in 25 mM sodium phosphate, pH 7.5, containing 2 mM CHAPS. The sephadex-reacted chlorin e6 mixture is allowed to react at room temperature for 20 minutes. The mixture is centrifuged at 1000 X g for 10 minutes. The un-reacted chlorin e6 in the mixture binds to the QAE-sephadex and is removed from the system via centrifugation. The modified chlorin e6 in the resulting supernatant is removed and added to transferrin immobilized to QAE-sephadex , wherein the conjugate forms on the sephadex. The bottom of Figure 1B shows a schematic of the chlorin *e*6-transferrin conjugate. With this procedure, non EDC-reacted chlorin e6 will retain a net negative charge and will bind to the QAE-sephadex. Chlorin *e*6 which has reacted with the EDC at two or more carboxyls will possess a net positive charge and will not bind to the QAE-sephadex. Therefore, only modified chlorin e6 will be added to the protein and non-specific adherence of chlorin e6 to the QAE-sephadex-transferrin will be avoided. The formation of the conjugate on the sephadex and elution from the agarose are performed as above..

ADDITIONAL PROCEDURE FOR THE REMOVAL OF FREE CHLORIN e6 USING CATION EXCHANGE IMMOBILIZATION:

The pooled chlorin e6-transferrin (Ce6Tf) is dialyzed at 4° C against 25 mM sodium acetate, pH 4.8. To eliminate remaining un-conjugated chlorin e6, the dialysate is combined with 2 ml of packed sulfopropyl-sepharose, previously equilibrated in the same buffer. This is mixed for 30 min at 25° C and the gel is washed three times by centrifugation from and re-suspension in 20 ml of the same equilibration buffer. This forms cation-immobilized chlorin *e*6-transferrin. The bound chlorin e6-transferrin is

released from the gel with 25 mM sodium phosphate, pH 7.2 ,containing 1.0 M NaCl. The released material is combined with 1/100 volume of 1% (w/v) ferric ammonium citrate, and dialyzed against 25 mM NaH2PO4, pH 7.2. With this procedure, transferrin possesses a net positive charge at a pH of 4.8, whereas un-modified (free) chlorin e6 retains a net negative charge. Therefore, the transferrin will bind to a negatively charged matrix, and the free chlorin e6 will not. This allows for the removal of free chlorin e6 via the washing procedure.

DEMONSTRATION OF THE ABILITY OF CHORIN *e*6-TRANSFERRIN TO INDUCE CELL GROWTH, FIGURE 2:

The effect of chorin e6-transferrin on the growth of Rat MTLn3 mammary adenocarcinoma cells is shown in Figure 2. Cells were plated at 2,000 cells/well in 96 well plates in 100 μL of ∝MEM containing 5% fetal bovine serum (FBS). One day after plating, media was changed to 100 μL ∝MEM containing 0.3% FBS. Increasing levels of human holo-transferrin (Native Tf) or human Ce6-transferrin (both in phosphate buffered saline = PBS) were added to respective wells, in the amount indicated. Four days later, cells were enumerated using a crystal violet stain assay, where Absorbance at 590 ηm correlates with cell number. Figure 2A shows an image of the crystal violet stained plate used in the assay. Figure 2B shows a plot of the absorbances from the plate shown in Figure2A. Cell number is seen to rise as the cells are exposed to increasing levels of native human Tf. A similar, albeit slightly lower rise was seen with Ce6-Tf, indicating intact biological activity in the latter.

PHYSICAL COMPARISON OF CHLORIN *e*6-TRANSFERRIN AND TRANSFERRIN BY ELECTROPHORETIC ANALYSIS, FIGURE 3:

The acrylamide gel solution consisted of 0.37 M Tris, 0.17 M HCl, 9.75 % w/v acrylamide, 0.25 % w/v Bis-acrylamide, 2 mM CHAPS, 0.01% v/v TEMED and 0.025% w/v ammonium persulfate. This was poured into a 15 X 15 X 0.1 cm chamber and allowed to polymerize. Proteins to be analyzed were dissolved at 100 μg/ml in PB. Pure preparations of bovine serum albumin (BSA), ovalbumin, and carbonic anhydrase were obtained from commercial sources, for use as standards. To 100 μl of each sample was added 33 μl of 1.48 M Tris, 0.68 M HCl, 8mM CHAPS, 0.01 % w/v bromophenol blue, and 20% v/v glycerol. Samples were loaded onto the acrylamide gel and the gel was placed into an electrophoresis chamber containing an anolyte of 20.16 M Tris, 0.01 N HCl. A catholyte of 0.02 M glycine and 0.01 N KOH was overlaid onto the gel and the samples were electrophoresed at 40 mA constant current until the dye front was 1 cm from the bottom of the gel. The gel was fixed in 40 methanol, 10% acetic acid and was stained in fixative containing 0.2% Coomassie blue R250. The gel was de-stained with repeated changes of fixative. The results are shown in Figure 3, where the migration pattern of two different preparations of chlorin e6-transferrin (Ce6-transferrin; Figure 3, Lanes 5 and 6) are shown to be different than that of native un-altered transferrin (Transferrin; Figure 3, Lane 4). The results indicate a greater mobility of chlorin e6-transferrin when compared to native transferrin.

DEMONSTRATION OF THE ABILITY OF CHLORIN *e*6-TRANSFERRIN TO COMPETE WITH FITC-TRANSFERRIN FOR BINDING TO CELL SURFACES, FIGURE 4:

Chlorin e6-transferrin was assessed for its to inhibit the binding of fluorescein conjugated-transferrin (FITC-Tf) to cell surfaces. FITC-Tf bound to the cells is detected by Western blotting of cell lysates and specific antibody-based detection of FITC in those. Rat MTLn3 mammary adenocarcinoma cells were grown to confluence in 12 well plates using media consisting of ∝MEM containing 5% v/v fetal bovine serum (FBS). Media was changed to ∝MEM only for 2 h an then again for overnight. The cells were equilibrated to 4° C, wells were drained and 1 ml of a binding buffer consisting of ∝MEM containing 25 mM HEPES (pH 7.5) and 3 mg/ml liquid gelatin was added to all wells. C*e*6-TF to be tested was added to respective wells to a final concentration of 1mg/ml. Native un-altered holo human transferrin, as a known control inhibitor, was added to positive control wells to a concentration of 1 mg/ml. Negative control wells received transferrin buffer only. FITC-Tf was added to control and test wells to a concentration of 100 μg/ml. Cells were incubated at 4° C for 2h. All wells were washed 4 times with 2 ml PBS and cells were lysed with 0.5 ml PBS containing 2% Triton X-100, 0.1 U/ml aprotinin, and 100 μg/ml PMSF. Lysate protein was determined using a BCA assay. Equal protein amounts of cell lysates were treated with SDS-PAGE treatment solution, were separated by SDS-PAGE, and blotted onto a nitrocellulose membrane. The blot was blocked and FITC-TF was detected by treatment of the blot with rabbit anti-FITC then with horseradish peroxidase (HRP) conjugated anti-rabbit

IgG. Membrane localized HRP was detected by enhanced chemiluminescence (ECL) using an HRP substrate. The results are shown in Figure 4, where an image of the resulting ECL X-ray film is shown. Figure 4, lanes 4 and 5 show a strong band at 70,000 Kd from cell lysates which received the FITC-Tf only, indicating binding of the FITC-Tf to the cells. Figure 4 lanes 6 and 8 show an absence of any FITC signal in lysates from cells treated with FITC-Tf, and either Ce6-Tf (Figure 4 lane 6), or native un-altered Tf (Figure 4 lane 8), thus indicating that both Ce6-Tf and Tf compete for the binding of FITC-TF to cell surfaces. Figure 4 lanes 1-3 show the results obtained when the gel lanes were loaded with known amounts of pure FITC-Tf, for standardization. The results indicate competition of the binding of FITC-Tf by Ce6-Tf, and thus show functional binding of Ce6-Tf to the transferrin receptor.

~~*Growth assays:* Rat MTLn3 mammary adenocarcinoma cells were plated at 2,000 cells/well in 96 well plates in ∝MEM containing 5% FBS. One day after plating, media was changed to ∝MEM containing 0.3% FBS. Increasing levels of human holo-Tf or human Ce6-Tf (both in PBS) were added to respective wells. Four days later, cells were quantitated using a crystal violet stain assay.~~

~~*Cell killing assays:* A: Serum-free media assays. These were performed to initially assess the effect of *Ce6*-Tf and to verify its light-dependent killing. Target cells were grown to confluence in 24-well plates. On the day of the assay, media in all wells was replaced with 1 ml of fresh serum-free media and increasing levels of *Ce6*-Tf added to~~

~~test wells. Native human holo-Tf was added, at the highest Ce6-Tf dose, to control wells.~~

~~B: Serum-containing, one day exposure assays. For these, serum was maintained, to emulate *in vivo* conditions where excess endogenous normal transferrin would be present. In addition, the Ce6-Tf exposure was limited to 1 day to emulate a one time Ce6-Tf injection. Target cells were grown to confluence in 24 well plates. On the day of the assay, media in all wells was replaced with 1 ml of fresh serum-containing media and increasing levels of Ce6-Tf added to test wells. Native human holo-Tf was added, at the highest Ce6-Tf dose, to control wells. One day after Tf addition, media in all plates was changed to normal culture media (without Ce6Tf).~~

~~With both assay methods, two plates for each line to be tested were plated and treated identically. One day after Ce6-Tf addition, test plates were exposed to the light from an X-ray film box for 15 min. : the box was placed horizontally and the culture plates placed directly on the cover glass. The parallel plate from a given line was kept in the dark. The light treatment was repeated for 3 days. Media was changed (with [A] or without [B] added Ce6-Tf) each day, to maintain cell viability. On the fourth day, the cells were quantitated using a crystal violet stain assay: wells were drained and washed 4 times with 2 ml PBS; cells were fixed with 1 ml 5% v/v glutaraldehyde (in PBS) at 25° C for 20 min.; wells were washed 4 times with 2 ml distilled water and stained with 1 ml of a 1:1 (v:v) mixture of 0.2% (w/v) crystal violet and 100 mM CAPS (pH 9.0). Wells were drained and washed 4 times with 2 ml distilled water. After drying, cell density was~~

~~determined using a Bio-Rad Multiimager, where ODU/mm2 correlates with cell number~~.

LIGHT-INDUCED CELL KILLING ASSAYS:

For light treatment, an X-ray box was placed horizontally and the culture plates placed directly on the X-ray box cover glass. For cell enumeration, cells were quantitated using a crystal violet stain assay, where tissue culture wells were drained and washed 4 times with 2 ml PBS; cells were then fixed by the addition of 1 ml 5% v/v glutaraldehyde (in PBS). Wells were incubated at 25° C for 20 min.; wells were washed 4 times with 2 ml distilled water and stained with 1 ml of a 1:1 (v:v) mixture of 0.2% (w/v) crystal violet and 100 mM 3-[cyclohexylamino]- 1-propanesulfonic acid, pH 9.0. Wells were then drained and washed 4 times with 2 ml distilled water, and allowed to dry. After drying, cell density was determined using a CCD camera equipped imager, where ODU/mm2 correlates with cell number.

DEMONSTRATION OF THE ABILITY OF CHLORIN *e*6-TF TO KILL RAT CELLS IN A SERUM-FREE ASSAY, FIGURE 5:

This was a continuous exposure, serum-free assay. Rat MTLn3 mammary adenocarcinoma cells were plated in 24 well plates and grown to confluency in ∝MEM containing 5% v/v FBS. On day one, media was changed to ∝MEM only and increasing levels of Ce6-Tf were added to test wells to a final concentration from 1.25 to 5.0 μg/ml. Native Tf was added to control wells at 5.0 μg/ml. On days 2, 3, and 4, cells were

exposed to light from an X-ray film box for 15 min. Media and all Tf was changed each day. On day five, all cells were quantitated using the crystal violet stain assay. Images of the stained plates are shown in Figure 5A. Stained cell numbers were evaluated using a CCD camera equipped imager. The results of image analysis are shown in Figure 5B, where ODU/mm2 correlates with cell number. Results indicate a light-dependent killing as plates kept in the dark during the process displayed no loss of cell numbers.

DEMONSTRATION OF THE ABILITY OF CHLORIN *e*6-TRANSFERRIN TO KILL RAT CELLS IN A SERUM-CONTAINING ASSAY, FIGURE 6:

For these, serum was maintained, to emulate *in vivo* conditions where excess endogenous normal transferrin would be present. In addition, the Ce6-Tf exposure was limited to 1 day to emulate a one time Ce6-Tf injection. Target cells consisting of rat MTLn3 mammary adenocarcinoma cells or normal rat kidney (NRK) fibroblast cells were grown to confluence in 24 well plates in a media of ∝MEM containing 5% v/v FBS. On the day of the assay, media in all wells was replaced with 1 ml of fresh media and increasing levels of Ce6-Tf added to test wells to a final concentration from 7.5 to 30 μg/ml.. Native human holo transferrin was added, at the highest Ce6-Tf concentration, to control wells. Cultures were returned to the incubator and one day after Tf addition, media in all plates was changed to normal culture media (without Ce6Tf or Tf). On days 2, 3, and 4, cells were exposed to light from an X-ray film box for 15 min. Media was changed each day. On day five, all cells were fixed, stained, and quantitated using the crystal violet stain assay. Stained cell numbers were evaluated using a CCD camera-

equipped imager. Images of the stained plates are shown in Figure 6A and Figure 6B. The results of image analysis are shown in Figure 6A and Figure 6D, where ODU/mm2 correlates with cell number. Results indicate a light-dependent killing as plates maintained in the dark during the process displayed no loss of cell numbers. The MTLn3 cell line was more susceptible to the effects of the Ce6-Tf as it showed a decrease in cell numbers at the 15 ug/ml dose whereas the normal NRK line did not.

DEMONSTRATION OF THE ABILITY OF CHLORIN *e*6-TRANSFERRIN TO KILL HUMAN CANCER CELLS IN A SERUM-CONTAINING ASSAY, FIGURE 7:

Human MCF7 breast cancer cells were plated in 24 well plates and grown to confluency in a media of ∝MEM containing 5% v/v FBS. On day one, media was changed and increasing levels of Ce6-Tf were added to test wells to a final concentration from 7.5 to 30 μg/ml. Native Tf was added to control wells at 30 μg/ml. On day 2, media in all wells was changed to that without added Ce6-Tf or Tf. On days 2, 3, and 4, cells were exposed to light from an X-ray film box for 15 min. Media was changed each day. On day five, all cells were fixed, stained, and quantitated using the crystal violet stain assay. Stained cell numbers were evaluated using a CCD camera-equipped imager. Images of the stained plates are shown in Figure 7A. The results of image analysis are shown in Figure 7B, where ODU/mm2 correlates with cell number. Results indicate a light-dependent killing as plates kept in the dark during the process displayed no loss of cell numbers. As with the rat lines studied previously, this human line was also shown to be susceptible to a combination of Ce6-Tf and light.

~~*Effect of Ce6 alone:* To determine if Ce6 alone, if added in appropriate concentrations, would induce cell death. Gel filtration analysis indicated no significant change in Tf's molecular weight after Ce6 conjugation (data not shown). It was assumed from this that less than 10 molecules of Ce6 were conjugated to each Tf protein. Ce6Tf was very active in causing light-induced cell death when initially present at 0.43 uM (30ug/ml), so free Ce6 was added to cultures at 4.3 uM, a ten fold molar excess, to ensure that Ce6 was present in greater amounts than that encountered by cells when exposed to Ce6Tf. So Ce6 was added to a final concentration of 2.5 μg/ml to confluent MTLn3 cells. Light-induced killing assays were conducted as stated in method B above.~~

DEMONSTRATION OF THE ABILITY OF CHLORIN *e*6 ALONE TO KILL CELLS VIA A LIGHT-INDUCED PROCESS, FIGURE 8:

To determine if Ce6 alone, if added in appropriate concentrations, would induce cell death. Ce6Tf is very active in causing light-induced cell death when initially present at 0.43 uM (30 μg/ml). Analysis of Ce6-Tf indicate that less than 10 molecules of Ce6 were conjugated to each Tf protein, so free Ce6 was added to cultures at 4.3 μM, a ten fold molar excess, to ensure that Ce6 was present in greater amounts than that encountered by cells when exposed to Ce6Tf. So Ce6 was added to a final concentration of 2.5 μg/ml to confluent MTLn3 cells. Light-induced killing assays were conducted using the serum containing method: Rat MTLn3 cells were grown to confluence in 24 well plates in a media of ∝MEM containing 5% v/v FBS. On the day of the assay, media

in all wells was replaced with 1 ml of fresh media and Ce6-Tf was added to test wells to a final concentration of 30 μg/ml. Certain wells received 2.5 μg/ml of Ce6 in place of Ce6-Tf. For a control, at the time of Ce6Tf addition, certain wells received human holo-transferrin so that the final concentration was also 30 μg /ml. Cultures were returned to the incubator and one day after Tf or Ce6 addition, media in all plates was changed to normal culture media (without Ce6Tf,Tf, or Ce6). On days 2, 3, and 4, cells were exposed to light from an X-ray film box for 15 min. Media was changed each day. On day five, all cells were fixed, stained, and quantitated using the crystal violet stain assay. Stained cell numbers were evaluated using a CCD camera-equipped imager. Images of the stained plates are shown in Figure 8A. The results indicate that Ce6 alone had no cell killing effect, when used at a concentration representative of ten times that present in the active dose of Ce6-Tf.

~~*Transferrin competition of cell killing:* These were performed to ensure that Ce6-Tf's cell killing effect was due to the function of the transferrin ligand: that the light-induced killing effect could be neutralized with excess native Tf. Method B. from above was used. Confluent cultures of MTLn3 cells in 24 well plates were treated with 30 ug/ml of Ce6Tf. At the time of Ce6Tf addition, certain wells also received human holo-transferrin so that the final concentration was 0.5 or 1.0 mg/ml. One day later, media was changed to normal culture media. Light-induced killing assays were continued and cells quantitated as stated above in method B.~~

DEMONSTRATION OF THE ABILITY OF NATIVE TF TO NEUTRALIZE LIGHT-INDUCED CHLORIN e6-TRANSFERRIN-MEDIATED CELL KILLING, FIGURE 8:

These were performed to ensure that Ce6-Tf's cell killing effect was due to the function of the transferrin ligand: that the light-induced killing effect could be neutralized with excess native Tf. The serum containing assay was used. Rat MTLn3 cells were grown to confluence in 24 well plates in a media of ∝MEM containing 5% v/v FBS. On the day of the assay, media in all wells was replaced with 1 ml of fresh media and Ce6-Tf was added to test wells to a final concentration of 30 μg/ml. At the time of Ce6Tf addition, certain wells also received human holo-transferrin so that the final concentration was 0.5 or 1.0 mg/ml. Cultures were returned to the incubator and one day after Tf addition, media in all plates was changed to normal culture media (without Ce6Tf or Tf). On days 2, 3, and 4, cells were exposed to light from an X-ray film box for 15 min. Media was changed each day. On day five, all cells were fixed, stained, and quantitated using the crystal violet stain assay. Stained cell numbers were evaluated using a CCD camera-equipped imager. Images of the stained plates are shown in Figure 8B. The results indicate that excess native Tf diminished the killing effect of Ce6-Tf, indicating that the latter acts through a Tf-specific process.

ABSTRACT OF THE DISCLOSURE:

The invention describes the synthesis and proposed usage of a tumor-specific, site-specific tumor cell-killing agent. The agent binds to tumor cells with high affinity and at the same time will bind minimally to surrounding normal cells. The agent has conjugated to it a porphyrin, which when exposed to light, generates cell-killing reactive oxygen species. Thus, in areas which can be irradiated by light, a site-specific, tumor-specific cell killing can occur. The agent consists of the iron-transport protein transferrin (Tf) which is conjugated with the porphyrin chorin *e*6 (Ce6). For this patent, a novel method of conjugation was developed as conventional methods of conjugation of chlorin *e*6 to the protein resulted in the loss of transferrin's biological activity. The new conjugation procedure results in the covalent attachment of chlorin e6 to transferrin and yet maintains the natural activity of the protein. The synthesis occurs while the protein is immobilized to QAE-sephadex, in the presence of the zwitterionic detergent CHAPS (3-[(3-cholidamidopropyl) dimethylammonio]- 1-propanesulfonate). Using this technique, the biological activity of the conjugated transferrin is preserved, the conjugate binds to cell surface transferrin receptors and promotes the growth of cells in culture, all while carrying the cell-killing chlorin *e*6. The conjugate induces a light-exposure dependent killing of tumor cells in tissue culture. After injection into cancer patients, a tumor cell killing effect will hypothetically be achieved by irradiation of the tumor site with light. The patent covers the new-found synthesis technique for and the *in vitro* and *in vivo* tumor cell killing usage of chlorin *e*6-transferrin.